Expression of Protease-Activated Receptor-2 in SZ95 Sebocytes and its Role in Sebaceous Lipogenesis, Inflammation, and Innate Immunity

Sang E. Lee[1], Ji-Min Kim[1], Se K. Jeong[2], Eung H. Choi[3], Christos C. Zouboulis[4] and Seung H. Lee[1]

Protease-activated receptor-2 (PAR-2) functions as innate biosensor for proteases and regulates numerous functions of the skin. However, the expression and physiological role of PAR-2 in sebocytes remain to be elucidated. Here, we identified PAR-2 expression in SZ95 sebocytes at both mRNA and protein levels. Intracellular Ca^{2+} mobilization by PAR-2 agonist peptide (PAR-2 AP) or *Propionibacterium acnes* (*P. acnes*) culture supernatant was detected, indicating that *P. acnes* is a potent activator of PAR-2 on sebocytes. The small interfering RNA (siRNA)–mediated PAR-2 knockdown in sebocytes resulted in defective differentiation and lipogenesis. PAR-2 AP treatment enhanced lipogenesis and sterol response element-binding protein-1 (SREBP-1) expression, suggesting a role of PAR-2 in the differentiation and lipogenesis of sebocytes. Moreover, PAR-2 AP induced cytokines and human β-defensin-2 (hBD-2) transcription in sebocytes. PAR-2 expression was increased in sebaceous glands of acne lesions. PAR-2 silencing by siRNA abrogated the increase in sebaceous lipogenesis and SREBP-1 expression by *P. acnes* supernatant. PAR-2 knockdown also inhibited the *P. acnes* supernatant–induced expression of cytokines and hBD-2. In conclusion, PAR-2 is expressed in SZ95 sebocytes and mediates differentiation, lipogenesis, inflammation, and innate immunity in response to *P. acnes*. Therefore, PAR-2 might be a therapeutic target for sebaceous gland disorders such as acne.

Journal of Investigative Dermatology (2015) **135,** 2219–2227; doi:10.1038/jid.2015.151; published online 14 May 2015

INTRODUCTION

The skin is exposed to various endogenous and exogenous proteases that have a role in skin physiology and pathophysiology (Meyer-Hoffert, 2009). Certain serine proteases, such as tryptase, kallikrein (KLK)-5, -6, and -14, or matriptase, and various pathogen-derived proteases activate protease-activated receptor-2 (PAR-2) in the skin, regulating permeability barrier homeostasis, keratinocyte cornification, pruritus, inflammation, pigmentation, and wound healing (Rattenholl and Steinhoff, 2003, 2008; Hachem *et al.*, 2006; Demerjian *et al.*, 2008).

Notably, previous studies have revealed that KLKs are not only expressed in the interfollicular epidermis but also in the pilosebaceous unit. Pro-KLK5 and KLK7 were identified in the sebaceous ducts, suggesting a role in the desquamation-like process at the pilosebaceous canal and sebaceous glands (Ekholm *et al.*, 1998, 2000). In another study, mRNA expression of KLK-4, -6, -9, -10, and -11, and the serine protease inhibitor Kazal-type 5 (SPINK5), was demonstrated in the basal and inner mature cells in sebaceous glands (Komatsu *et al.*, 2003). In addition, KLK-6, -8, and -13 were also found in sebaceous glands, especially in the cytoplasm of basal cells (Komatsu *et al.*, 2005). Coexpression of various KLKs and SPINK5 in sebaceous glands suggests that the balance between serine proteases and their inhibitor might regulate sebocyte functions and mediate the pathophysiology of diseases associated with sebaceous glands.

The sebaceous glands have a critical role in the pathogenesis of acne vulgaris (Makrantonaki *et al.*, 2011). In the lesional skin of acne, infiltration of activated mast cells (Toyoda and Morohashi, 2003) and neutrophils (Kang *et al.*, 2005) was detected in adjacent areas to the sebaceous glands, and these cells might produce proteases such as tryptase, leukocyte elastase, and cathepsin G that are able to target PAR-2 (Steinhoff *et al.*, 1999, 2000; Uehara *et al.*, 2003; Moormann *et al.*, 2006). Moreover, we have recently demonstrated that proteases produced by a certain strain of *Propionibacterium acnes* (*P. acnes*) activate PAR-2 on keratinocytes and induce inflammatory cytokines, antimicrobial peptides, and matrix metalloproteinases, implicating an involvement of PAR-2 signaling in acne pathogenesis (Lee *et al.*, 2010).

In human skin, PAR-2 is abundantly expressed by keratinocytes; however, the expression and function of

[1]Department of Dermatology and Cutaneous Biology Research Institute, Yonsei University College of Medicine, Seoul, Korea; [2]Research Division Neopharm, Daejeon, Korea; [3]Department of Dermatology, Yonsei University Wonju College of Medicine, Wonju, Korea and [4]Departments of Dermatology, Venereology, Allergology, and Immunology, Dessau Medical Center, Dessau, Germany

Correspondence: Seung H. Lee, Department of Dermatology, Yonsei University College of Medicine, Gangnam Severance Hospital, 712 Eonjuro, Gangnam-gu, Seoul 135-720, Korea. E-mail: ydshderm@yuhs.ac

Received 28 January 2015; revised 17 March 2015; accepted 7 April 2015; accepted article preview online 16 April 2015; published online 14 May 2015

PAR-2 in sebaceous glands have not been systematically investigated.

In this study, we identified the existence of functional PAR-2 in SZ95 sebocytes and explored the role of PAR-2-mediated signaling in sebaceous gland biology, such as sebocyte differentiation, lipid synthesis, inflammation, and innate immunity. In addition, we assessed whether *P. acnes*, which produces extracellular proteases, is a potent activator of PAR-2 in sebocytes via measuring Ca^{2+} signaling. Then, we explored whether PAR-2 on SZ95 sebocytes is involved in the recognition of *P. acnes* and mediates lipogenesis and the expression of inflammatory cytokines and antimicrobial peptides in response to *P. acnes*.

RESULTS

PAR-2 is expressed in the cultured human SZ95 sebocytes

First, we aimed to demonstrate the existence of PAR-2 in SZ95 sebocytes. To examine the localization of PAR-2 in SZ95 sebocytes, we used immunofluorescent confocal microscopy. Under basal conditions, PAR-2 was expressed at low levels in a granular pattern within the cytoplasm of SZ95 sebocytes. Combined treatment with 2×10^{-8} M testosterone and 10^{-4} M linoleic acid (T/LA) for 24 hours resulted in an increase in the fluorescence intensity of PAR-2, suggesting that PAR-2 expression increases upon sebocyte differentiation (Figure 1a). PAR-2 immunofluorescence was observed within the cytoplasm, mainly around the cell periphery in a punctate staining pattern (Figure 1a). PAR-2 expression on sebocytes at the protein level was confirmed by western blotting. By western blotting, PAR-2 expression was increased in T/LA-induced differentiated SZ95 sebocytes compared with undifferentiated sebocytes (Figure 1b). We next investigated the expression of PAR-2 at the gene level by real-time PCR using PAR-2-specific primers that amplified a product of 491 bp and real-time PCR. PAR-2 mRNA was detected in both undifferentiated and differentiated SZ95 sebocytes (Figure 1c). Real-time PCR data revealed that PAR-2 gene expression was significantly higher in the SZ95 sebocytes incubated with T/LA compared with the undifferentiated sebocytes (Figure 1d). Taken together, these data demonstrate the existence of PAR-2 in human SZ95 sebocytes at both mRNA and protein levels, with higher expression in more differentiated cells after T/LA treatment.

Knockdown of PAR-2 suppresses sebocyte differentiation

To explore whether PAR-2 has a functional role in sebocyte differentiation, we transfected SZ95 sebocytes with small interfering RNAs (siRNAs) that specifically blocked PAR-2 expression (PAR-2 siRNA) or scrambled siRNA and subsequently treated the cells with T/LA for 24 hours. First, we evaluated the efficiency of PAR-2 siRNA in SZ95 sebocytes. Figure 2a shows that the transfection of SZ95 sebocytes with PAR-2 siRNA significantly decreased PAR-2 gene expression by 63% and the scrambled siRNA, a negative control, had no effect, demonstrating the specificity of the PAR2 siRNA sequence. Western blot analysis showed that PAR-2 siRNA transfection effectively reduced PAR-2 protein expression (Figure 2b). Sebocyte differentiation was assessed by measuring the mRNA levels of melanocortin receptor 5 (MC5R) and epidermal membrane antigen, as these genes are expressed highly in the differentiated sebocytes (Zhang *et al.*, 2006; Zouboulis *et al.*, 2008). Knockdown of PAR-2 by PAR-2 siRNA resulted in significantly decreased MC5R and epidermal membrane antigen mRNA levels after T/LA treatment in comparison with cells transfected with a scrambled control (Figure 2c and d). Differentiation of sebocytes is accompanied by the sebaceous lipid synthesis. Therefore, we also investigated the effect of PAR-2 silencing on the T/LA-induced lipid synthesis. As previously reported, T/LA treatment enhanced the accumulation of lipid droplets and the synthesis of neutral lipids in SZ95 sebocytes (Figure 2e and f), reflecting stimulation of sebocyte differentiation (Wróbel *et al.*, 2003), whereas knockdown of PAR-2 by RNA interference significantly inhibited the T/LA-induced sebaceous lipid synthesis compared with scrambled siRNA-transfected controls (Figure 2e and f). Taken together, these data strongly suggest that PAR-2 signal exerts a critical role in sebocyte differentiation and lipid accumulation, a key step in the differentiation of sebocytes.

PAR-2 activation induces Ca^{2+} signaling in SZ95 sebocytes, and *P. acnes* culture supernatant-induced calcium transient in sebocytes is related to PAR-2 activation

To further determine the existence of functional PAR-2 on sebocytes, we investigated whether PAR-2 on SZ95 sebocytes is coupled to the intracellular calcium ($[Ca^{2+}]_i$) signaling. SZ95 sebocytes were loaded with Ca^{2+}-sensitive fluorescence dye fura 2 and stimulated with PAR-2 agonist peptide (PAR-2 AP). As shown in Figure 3a, the $[Ca^{2+}]_i$ in sebocytes increased rapidly with a peak at ~40–60 seconds after stimulation with PAR-2 AP in a dose-dependent manner. Repeated treatment with PAR-2 AP reduced subsequent responses to the same concentration of agonist peptide, suggesting that PAR-2 AP activates and selectively desensitizes PAR-2 in sebocytes (Figure 3b). PAR-2 AP–induced Ca^{2+} signaling was the same in Ca^{2+}-free solution as in normal solution, suggesting that PAR-2 AP–induced $[Ca^{2+}]_i$ increases were due to Ca^{2+} release from intracellular stores (Figure 3b and c).

We next investigated the ability of *P. acnes* to signal (calcium transient) via PAR-2 in SZ95 sebocytes. Similar increase in $[Ca^{2+}]_i$ was detected in SZ95 sebocytes after treatment with 2.5% concentration of *P. acnes* culture supernatant (Figure 3d). Repeated stimulation with *P. acnes* supernatant reduced subsequent responses (Figure 3e), suggesting desensitization of the calcium signal. Removal of extracellular Ca^{2+} and treatment with *P. acnes* supernatant also elicited a similar increase in $[Ca^{2+}]_i$ in sebocytes (Figure 3e and f).

To confirm the PAR-2 dependence of *P. acnes* supernatant–mediated Ca^{2+} signaling, we used siRNA to knockdown PAR-2 expression. PAR-2 knockdown significantly decreased *P. acnes* supernatant–induced Ca^{2+} responses, whereas Ca^{2+} signaling persisted in scrambled siRNA-transfected controls (Figure 3g). These results indicate that SZ95 sebocytes express functional PAR-2 coupled to the intracellular calcium signaling, and secreted *P. acnes* proteases can activate PAR-2 on sebocytes.

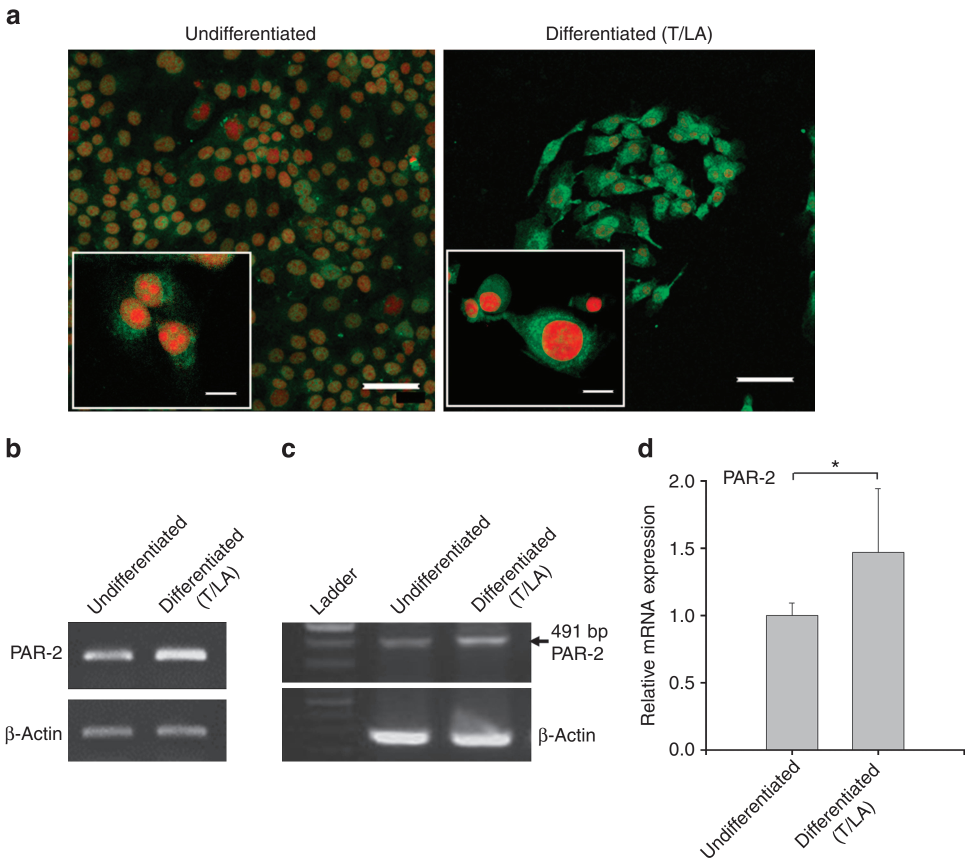

Figure 1. Protease-activated receptor-2 (PAR-2) expression and localization in SZ95 sebocytes. (**a**) Immunofluorescence staining of PAR-2 in the undifferentiated (UnD-SZ95) and differentiated SZ95 sebocytes (D-SZ95) induced by 24 hours of treatment with testosterone/linoleic acid (T/LA). Nuclei were counterstained by propidium iodide (red fluorescence). Scale bar = 50 μm. Insets of higher magnification show PAR-2 localization. Scale bar = 10 μm. (**b**) Western blot for PAR-2 in undifferentiated and T/LA-differentiated sebocytes. (**c**) Total RNA of SZ95 sebocytes was isolated, reverse transcribed, and subjected to PAR-2 real-time PCR (RT-PCR). Molecular markers were used to verify the sizes of RT-PCR products. β-Actin was used as an internal control. (**d**) The mRNA levels of PAR-2 were analyzed by RT-PCR in undifferentiated and T/LA-differentiated SZ95 sebocytes. Data are shown as mean ± SD of three independent experiments (**P* < 0.05).

PAR-2 expression is increased in sebaceous glands of acne lesions

To verify the expression of PAR-2 in sebaceous glands of acne lesions, the immunohistochemical evaluation of PAR-2 in normal human facial skin and inflammatory acne-involved facial skin samples was performed. Sebaceous glands of inflammatory acne lesions expressed strong membranous and intracytoplasmic staining of PAR-2 in almost all cells (Figure 4). In sebaceous glands of the normal facial skin, immunoreactivity of PAR-2 was less intense than in the biopsy samples of the acne patients (Figure 4). These findings suggest the possible involvement of PAR-2 signal in sebaceous glands in the pathogenesis of acne.

PAR-2 activation enhances lipid synthesis and SREBP-1 expression in SZ95 sebocytes

To further explore the physiologic role of PAR-2 signaling in sebocytes, we next aimed to investigate whether PAR-2 activation induces sebaceous lipid synthesis and cultured undifferentiated and T/LA-induced differentiated SZ95 sebocytes in the presence of PAR-2 AP or phosphate-buffered saline (vehicle control) for 24 hours. In undifferentiated sebocytes, PAR-2 AP did not significantly affect lipogenesis, whereas in T/LA-induced differentiated sebocytes stimulation with PAR-2 AP significantly increased lipid accumulation in the cytoplasm compared with vehicle controls when examined by microscopy after semiquantitative Oil Red O (Figure 5a) and quantitative Nile Red staining (Figure 5b).

To investigate the mechanism involved in the PAR-2 activation–induced sebaceous lipogenesis, we studied the effect of PAR-2 AP on the expression of sterol response element binding protein-1 (SREBP-1), the master regulator of lipid biosynthesis, in sebocytes. Western blot analysis showed that PAR-2 AP treatment significantly increased the expression of precursor and mature forms of SREBP-1 compared with vehicle controls but did not alter the mature/precursor SREBP-1 ratio (Figure 5d). In addition, real-time PCR demonstrated that the SREBP-1a and -1c mRNA transcripts were significantly increased in the sebocytes in response to PAR-2 AP (Figure 5e). These results suggest that SREBP-1 induction might be involved, at least in part, in the PAR-2 activation–induced lipogenesis in SZ95 sebocytes.

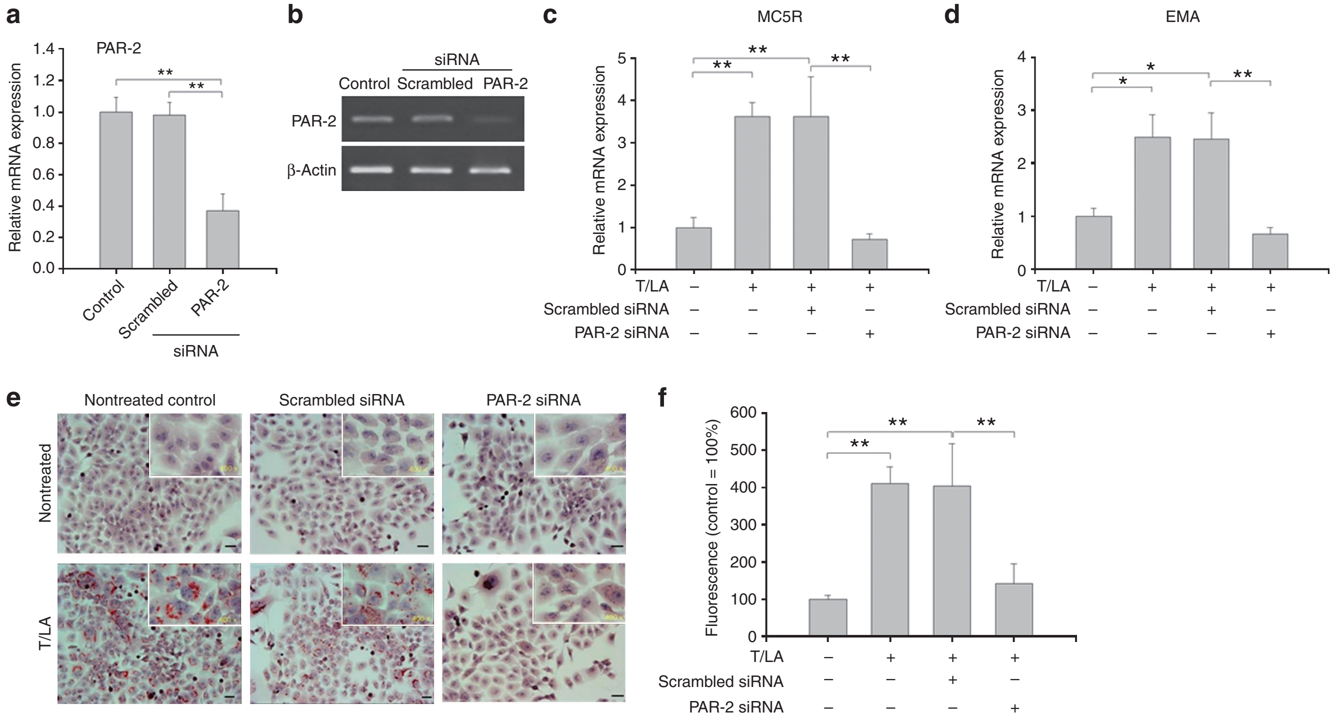

Figure 2. Protease-activated receptor-2 (PAR-2) silencing by small interfering RNA (siRNA) transfection suppresses testosterone/linoleic acid (T/LA)–induced sebocyte differentiation. SZ95 sebocytes were transfected with 50 nM PAR-2 siRNA or scrambled siRNA. At 24 hours after transfection, PAR-2 mRNA and protein expression was evaluated using (**a**) real-time PCR and (**b**) western blotting, respectively, and differentiation was induced by T/LA treatment for 24 hours. (**c** and **d**) The mRNA expression of sebocyte differentiation marker, melanocortin receptor 5 (MC5R), and epithelial membrane antigen (EMA) was measured by real-time PCR. (**e**) Lipid accumulation was evaluated by Oil Red O staining. Scale bars = 50 μm. (**f**) Quantitative analysis of lipid synthesis was assessed by Nile Red staining followed by fluorometric imaging plate reader (FLIPR) measurement. Values represent percentages of the controls and show the mean ± SD of three independent experiments (*$P<0.05$; **$P<0.01$).

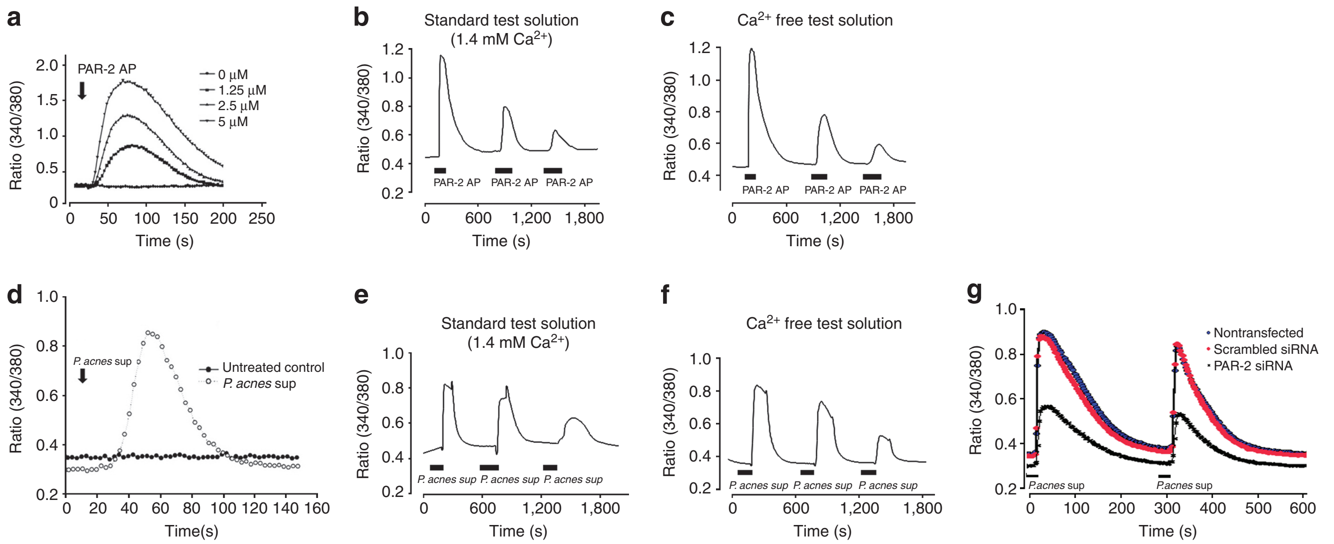

Figure 3. Intracellular calcium responses in SZ95 sebocytes stimulated with protease-activated receptor-2 agonist peptide (PAR-2 AP) or the culture supernatant of *Propionibacterium acnes*. SZ95 sebocytes were loaded with Ca^{2+} indicator fura-2-AM, and fluorescence measurements reflecting the elevations of $[Ca^{2+}]_i$ in response to various concentrations of (**a**) PAR-2 AP (1.25–5 μM) or (**d**) *P. acnes* supernatant (sup) were conducted using a fluorescence spectrometer. SZ95 sebocytes were desensitized with repeated treatment with (**b**) PAR-2 AP (2.5 μM) or (**e**) *P. acnes* sup. The $[Ca^{2+}]_i$ signal in response to (**b** and **c**) PAR-2 AP or (**e** and **f**) *P. acnes* sup in SZ95 sebocytes is shown in the presence and absence of 1.4 mM extracellular Ca^{2+}. (**g**) *P. acnes* sup–induced $[Ca^{2+}]_i$ signals of PAR-2 small interfering RNA (siRNA)–transfected and scrambled RNA-transfected sebocytes. Fluorescence ratio, 340 nm/380 nm.

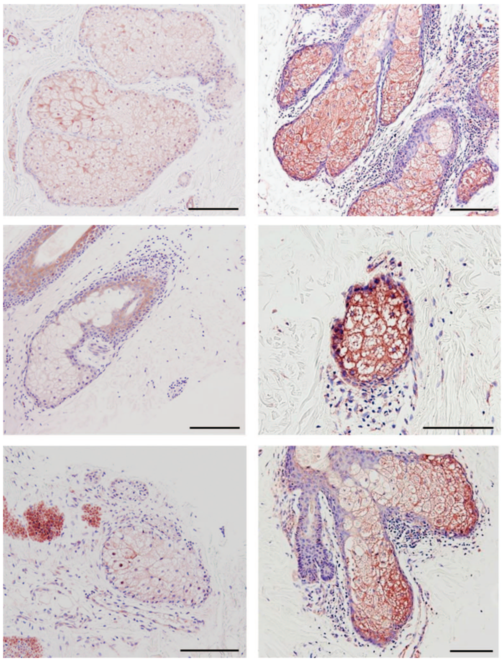

Figure 4. Expression of protease-activated receptor-2 (PAR-2) in the sebaceous glands of acne lesions. Paraffin-embedded tissues of normal human facial skin ($n=5$) and facial inflammatory acne lesions ($n=5$) were immunostained for PAR-2. Scale bars = 100 μm.

Knockdown of PAR-2 inhibits *P. acnes* culture supernatant-induced lipogenesis and SREBP-1 expression in SZ95 sebocytes

We demonstrated that the culture supernatant of *P. acnes* activated PAR-2 in sebocytes. Therefore, we next examined the involvement of PAR-2 signal in *P. acnes*–induced sebum synthesis and SREBP-1 expression by siRNA-mediated PAR-2 silencing. Untransfected and PAR-2 siRNA– or scrambled siRNA–transfected sebocytes were treated with T/LA to induce differentiation and then incubated with *P. acnes* culture supernatant or medium alone (control). *P. acnes* supernatant significantly increased lipid synthesis in SZ95 sebocytes compared with control (Figure 5a and c). *P. acnes* supernatant also enhanced the protein amount of SREBP-1 in both precursor and mature forms in SZ95 sebocytes (Figure 5d). The induction of lipid synthesis and SREBP-1 expression after *P. acnes* stimulation were significantly inhibited by PAR-2 silencing (Figure 5a, c, and d). These results suggest that *P. acnes*, which has protease activity, activates PAR-2 on sebocytes and induces lipogenesis partly via PAR-2 signal.

PAR-2 activation induces proinflammatory cytokines and hBD-2 transcription in SZ95 sebocytes, and *P. acnes*–induced expression of cytokines and hBD-2 is attenuated by PAR-2 silencing

To further assess a role of PAR-2 in sebaceous gland inflammation and innate immune response, we next investigated whether PAR-2 signaling is involved in the production of proinflammatory cytokines and antimicrobial peptides in SZ95 sebocytes. As shown in Figure 6a, significant induction of IL-8 and tumor necrosis factor-α (TNF-α) mRNA expression was observed in sebocytes stimulated with PAR-2 AP compared with vehicle controls. Human β-defensin-2 (hBD-2) mRNA level was significantly upregulated after stimulation with PAR-2 AP, whereas LL-37 transcript was not affected by agonist treatment (Figure 6d). These results indicate a role of PAR-2 in the regulation of inflammatory and innate immune response in sebaceous glands. Then, we examined the involvement of PAR-2 in *P. acnes*–induced cytokines and hBD-2 mRNA expression in sebocytes by siRNA-mediated knockdown of PAR-2. In line with the previous results, we found that *P. acnes* culture supernatant induced the expression of proinflammatory cytokines IL-8 and TNF-α in SZ95 sebocytes. In the transfected cells, *P. acnes* supernatant–induced IL-8 and TNF-α mRNA expression was significantly inhibited by PAR-2-specific siRNA (Figure 6b and c). In addition, PAR-2 siRNA inhibited *P. acnes*–induced increase in gene expression of hBD-2 in sebocytes (Figure 6e). These results suggest that *P. acnes*–induced production of proinflammatory cytokines and hBD-2 in sebocytes is partly mediated by PAR-2 signaling.

DISCUSSION

In this study, we identified that PAR-2 is present at the gene and protein levels in SZ95 sebocytes, an immortalized human sebaceous gland cell line. PAR-2 signals primarily by coupling to the heterotrimeric G proteins, leading to a transient increase in intracellular Ca^{2+}. We also demonstrated that PAR-2 AP triggers a transient rise in cytosolic Ca^{2+} in a dose-dependent manner and repeated stimulation of agonist peptide induces desensitization of $[Ca^{2+}]_i$ mobilization in SZ95 sebocytes, suggesting that PAR-2 is functionally active in human sebocytes.

Functional studies demonstrated a role of PAR-2 for sebocyte biology, including sebocyte differentiation, lipid synthesis, and the gene expression of proinflammatory cytokines and antimicrobial peptide.

The mRNA and protein levels of PAR-2 were higher in T/LA-differentiated sebocytes compared with those in undifferentiated cells, suggesting that increased expression of PAR-2 might be related to the differentiation status of sebocytes. However, to verify whether PAR-2 upregulation is specific for T/LA treatment or a general phenomenon in the process of sebocyte differentiation, further studies will be needed. In addition, we observed that siRNA-mediated silencing of PAR-2 significantly inhibited T/LA-induced expression of sebocyte differentiation markers and intracellular lipid accumulation. These data strongly suggest that PAR-2 signal has an important role in sebocyte differentiation and associated lipid synthesis, the major step of the sebaceous gland differentiation process. The involvement of PAR-2 signal in the regulation of the sebaceous lipid synthesis is further supported by the increased lipogenesis in the differentiated SZ95 sebocytes after stimulation with PAR-2 AP. Taken together, these data suggest that, under physiological conditions, certain extracellular proteases signal to sebocyte via PAR-2 activation that contributes to sebocytes differentiation. However, under pathologic conditions with enhanced local

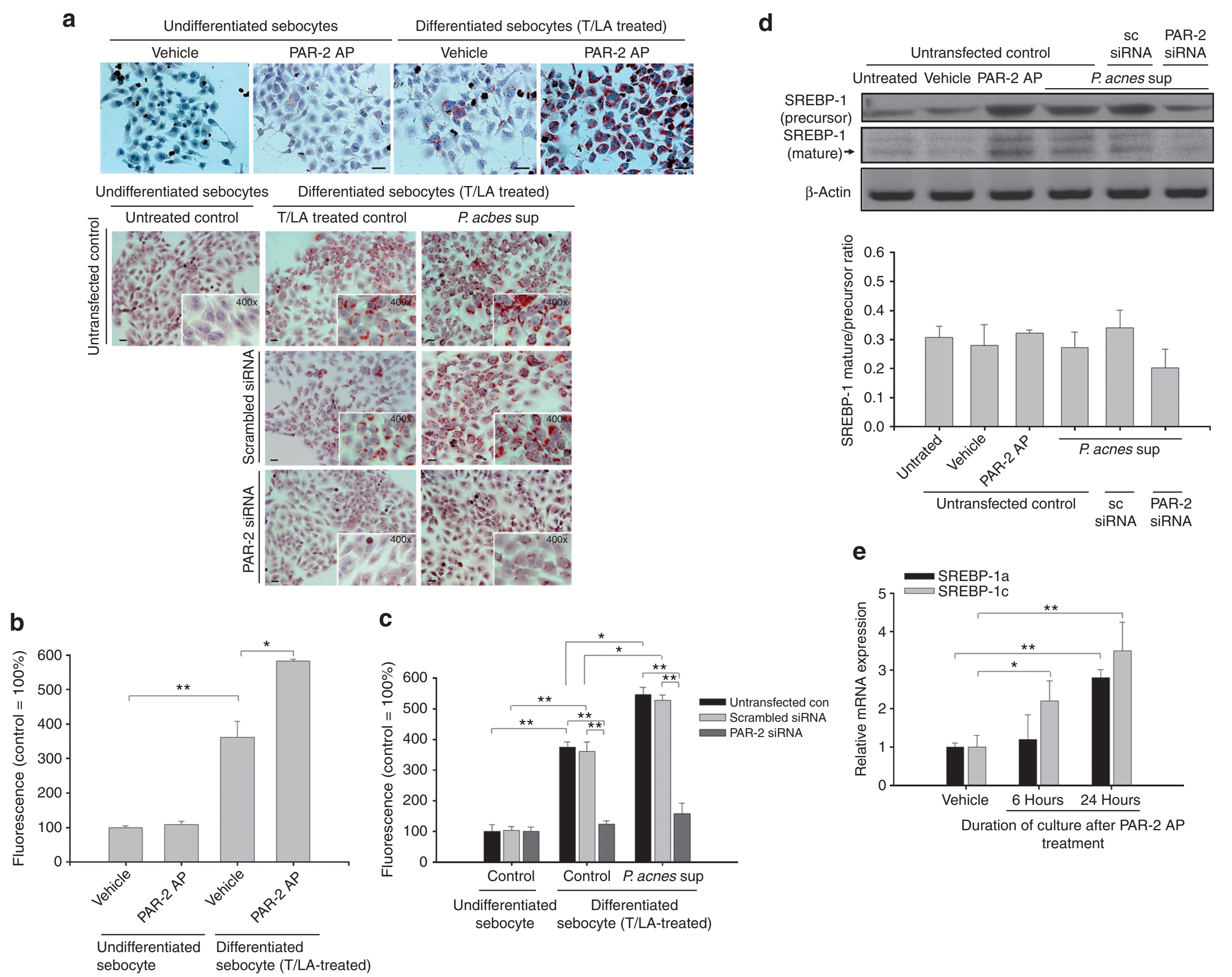

Figure 5. Protease-activated receptor-2 (PAR-2) activation by agonist peptide (AP) or the culture supernatant (sup) of *Propionibacterium acnes* induces lipogenesis and sterol regulatory element binding protein-1 (SREBP-1) expression in SZ95 sebocytes. Undifferentiated and testosterone/linoleic acid (T/LA)–differentiated SZ95 sebocytes were treated with PAR-2 AP (2.5 μM) or phosphate-buffered saline (PBS; vehicle control) for 24 hours. SZ95 sebocytes were transfected with scramble (sc) or PAR-2 small interfering RNA (siRNA). After induction of differentiation, *P. acnes* supernatant was added to the culture medium (2.5%) and incubated for 24 hours. Lipid accumulation was evaluated by (**a**) Oil Red O and (**b** and **c**) Nile Red staining. Scale bars = 50 μm. (**d**) Western blot analysis for SREBP-1 and quantification of the mature/precursor form ratio. (**e**) SREBP-1a and -1c mRNA levels were analyzed by real-time PCR. Data represent the mean ± SD of three independent experiments (*$P<0.05$; **$P<0.01$).

levels of pathogen- or host-derived proteases such as proteases from inflammatory infiltrates, sebocyte PAR-2 signaling may contribute to augment sebum production that is pivotal in the pathogenesis of acne.

In addition, we observed an increase in the SREBP-1 mRNA level and the amount of both precursor and mature forms of SREBP-1 protein in SZ95 sebocytes after PAR-2 AP treatment, suggesting that increased synthesis of SREBP-1 through PAR-2 signaling in sebocytes is a possible mechanism for the increase in sebaceous lipogenesis in response to PAR-2 activation. The SREBPs are key transcriptional factors that regulate genes encoding enzymes of cholesterol and fatty acid synthesis in sebaceous glands (Harrison *et al.*, 2007), and the SREBP pathway is known to be involved in the androgen and IGF-1-mediated sebum production (Rosignoli *et al.*, 2003; Smith *et al.*, 2008).

Previous studies have demonstrated that PAR-2 activation contributes to cutaneous inflammation (Steinhoff *et al.*, 2005). In this study, we observed that PAR-2 activation via agonist peptide significantly enhanced IL-8 and TNF-α gene expression by SZ95 sebocytes, suggesting a role of PAR-2 signal in mediating inflammatory responses in sebaceous glands. Furthermore, recent evidence suggests that sebaceous glands contribute to cutaneous immune defense by releasing antimicrobial peptides or sebum-free fatty acids (Nagy *et al.*, 2006; Nakatsuji *et al.*, 2010). Here, we demonstrated that PAR-2 AP stimulation induced hBD-2 transcription in SZ95 sebocytes, indicating that PAR-2 signaling also mediates innate immune responses in sebaceous glands.

Given our findings that PAR-2 mediates signals that enhance lipid synthesis and inflammatory responses in SZ95 sebocytes, we hypothesized that PAR-2 in sebaceous glands

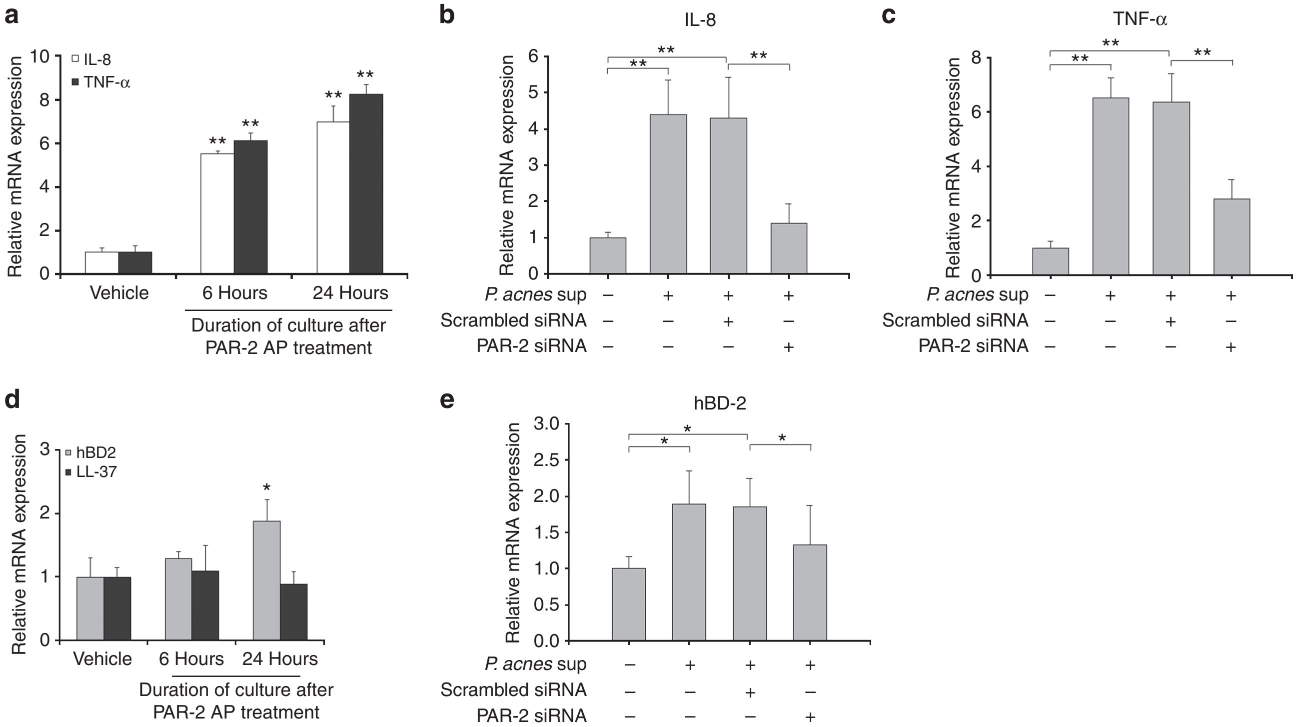

Figure 6. Protease-activated receptor-2 agonist peptide (PAR-2 AP) induces proinflammatory cytokines and human β-defensin-2 (hBD-2) transcription and *Propionibacterium acnes* supernatant–induced increases in cytokines and hBD-2 were abrogated by PAR-2-specific small interfering RNA (siRNA) transfection in SZ95 sebocytes. SZ95 sebocytes were treated with PAR-2 AP (2.5 μM) and the gene expression of (**a**) IL-8 and tumor necrosis factor-α (TNF-α) and (**d**) antimicrobial peptide (hBD-2 and LL-37) was analyzed using real-time PCR. (**b**, **c** and **e**) Sebocytes were transfected with PAR-2 siRNA or scrambled siRNA. At 24 hours after transfection, the medium was changed to complete medium with or without *P. acnes* supernatant at a final concentration of 2.5%. The mRNA expression of IL-8, TNF-α, and hBD-2 was evaluated 24 hours after stimulation using real-time PCR. Data are shown as mean ± SD of three independent experiments (*$P<0.05$; **$P<0.01$).

may have a role in the pathogenesis of acne and studied the expression of PAR-2 in sebaceous glands of inflammatory acne lesional skin and compared it with facial biopsies from healthy subjects. Our immunohistochemical data showed strong upregulation of PAR-2 in the acne-involved sebaceous glands compared with normal glands, implicating that increased PAR-2 expression in sebocytes may contribute to the pathogenesis of acne.

The exact endogenous activators of PAR-2 in sebocytes have not been identified yet. In this study, we demonstrated that the culture supernatant of *P. acnes* strain ATCC6919, which was demonstrated to have protease activity in a previous own study (Lee *et al.*, 2010), activates PAR-2 in SZ95 sebocytes by observing *P. acnes*–induced intracellular calcium mobilization and receptor desensitization. PAR-2 activation by *P. acnes* in sebocytes is further supported by the inhibition of *P. acnes*–induced Ca^{2+} signaling in SZ95 sebocytes after PAR2 siRNA transfection.

In addition, increased infiltration of activated mast cells and neutrophils has been reported in acne lesional skin. Therefore, in the inflammatory acne lesions, PAR-2 signaling in sebocytes may be regulated by various possible activators including *P. acnes*–secreted proteases, as well as mast cell tryptase or neutrophil proteases (Uehara *et al.*, 2003; Moormann *et al.*, 2006).

Recent studies suggest that the activation of innate immunity in keratinocytes and sebocytes by *P. acnes* and subsequent proinflammatory responses are major factors for the inflammatory reaction in acne vulgaris. Toll-like receptor and PAR-2 represent two distinct receptors related to innate immunity. Although Toll-like receptor-2–mediated cytokine expression by *P. acnes* in sebocytes is known to be involved in acne pathogenesis, the role of PAR-2 in mediating immune response to *P. acnes*–secreted proteases has not been addressed. Herein, we demonstrated that *P. acnes* culture supernatant-induced IL-8 and TNF-α gene expression is mediated by PAR-2 by observing a significant decrease in *P. acnes*–induced upregulation of these inflammatory cytokines in PAR-2 siRNA–transfected sebocytes. In addition, we noted that *P. acnes*–induced upregulation of hBD-2 occurred via PAR-2. Certain phylogenetic groups of *P. acnes* are pathogenic and may aggravate acne, but *P. acnes* is normally a commensal bacterium that modulates the production of antimicrobial peptides and inflammatory mediators, allowing the skin to develop a protective certain level of innate immunity (Zouboulis, 2009; Alexeyev *et al.*, 2012). These findings suggest that, under normal conditions, *P. acnes*/PAR-2 signaling does not exert pathological effects and may have a role in enhancing innate immunity and maintaining homeostasis.

We also demonstrated that certain strain of *P. acnes* participates in the enhancement of sebum production in sebocytes through PAR-2 signaling. The role of *P. acnes* in the sebaceous lipogenesis is still unclear (Zouboulis, 2009), but a recent study demonstrated that *P. acnes* extract and formalin-killed *P. acnes* (strains JCM 6473 and 6425) induced lipogenesis in hamster sebaceous glands through an increase in 15-deoxy-Δ12,14-prostaglandin J2 production (Iwata *et al.*, 2005). In this study, we observed that *P. acnes* supernatant with protease activity increased lipid synthesis in sebocytes and PAR-2 silencing by siRNA significantly inhibited the *P. acnes* supernatant–induced increase in lipogenesis and SREBP-1 expression in SZ95 sebocytes. These findings suggest that PAR-2 signaling is involved in and is at least partly responsible for the enhancement of sebum production in sebocytes in response to *P. acnes*.

In conclusion, we demonstrate that functional PAR-2 is expressed in SZ95 sebocytes and mediates sebocyte differentiation, lipogenesis, inflammation, and innate immunity. This study also shows that *P. acnes* extracts activate PAR-2 in SZ95 sebocytes and induce lipid synthesis and the expression of proinflammatory cytokines and hBD-2 in sebocytes via PAR-2 signaling. These data strongly suggest that PAR-2 regulates important physiological functions of sebocytes, and the modulation of PAR-2 signaling might be a promising therapeutic target for the treatment of disorders involving the sebaceous glands such as acne vulgaris.

MATERIALS AND METHODS

Further details are available in Supplementary Materials and Methods online.

Reagents and bacterial extracts

Human PAR-2 AP (SLIGKV-NH_2) was purchased from Peptron (Daejeon, Korea) and dissolved in phosphate-buffered saline. *P. acnes* (ATCC 6919, Manassas, VA) was grown in the brain heart infusion broth (Difco, Sparks, MD) at 37 °C for 24 hours under anaerobic condition to log phase. Bacterial cultures were centrifuged at $5{,}000 \times g$ for 15 minutes. The supernatants were harvested, filtered through a 0.2-μm pore size filter, and then stored at −20 °C until used.

Cell culture and differentiation induction

The immortalized human SZ95 sebaceous gland cell line (Zouboulis *et al.*, 1999) was cultured in DMEM/F-12 supplemented with 2 mM glutamax I, 10 μg ml^{-1} gentamicin, 50 ng ml^{-1} human EGF, 10% fetal bovine serum, and 10 mM HEPES (all from Gibco BRL, Rockville, MD). Cells were treated with the combination of 10^{-4} M linoleic acid and 2×10^{-8} M testosterone for 24 hours or maintained for 24 hours in the medium. T/LA treatments induced cytoplasmic lipid accumulation and the expression of epithelia membrane antigen, suggesting the differentiation of sebocytes. Cells were maintained at 37 °C/5% CO_2 up to the third passage level, and the medium was replaced every 2 days.

PAR-2 knockdown by RNA interference

For PAR-2 knockdown study, SZ95 sebocytes were transfected by addition of transfection cocktail containing 50 nM PAR-2 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. As a control, 50 nM siRNA control sequence was transfected in the same procedure. The PAR-2 siRNA construct was obtained as ON-TARGET plus SMART pool PAR2 (L-005095-00-0005), and the nontargeting siRNA control was obtained as ON-TARGET plus siCONTROL nontargeting pool (D-001810-10-05) from Dharmacon (Thermo Fisher Scientific, Lafayette, CO). Cells were incubated for 24 hours at 37 °C, and the efficiency of PAR-2 knockdown was evaluated by real-time PCR and western blotting.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alexeyev OA, Lundskog B, Ganceviciene R *et al.* (2012) Pattern of tissue invasion by Propionibacterium acnes in acne vulgaris. *J Dermatol Sci* 67:63–6

Demerjian M, Hachem JP, Tschachler E *et al.* (2008) Acute modulations in permeability barrier function regulate epidermal cornification: role of caspase-14 and the protease-activated receptor type 2. *Am J Pathol* 172: 86–97

Ekholm E, Sondell B, Strandén P *et al.* (1998) Expression of stratum corneum chymotryptic enzyme in human sebaceous follicles. *Acta Derm Venereol* 78:343–7

Ekholm IE, Brattsand M, Egelrud T. (2000) Stratum corneum tryptic enzyme in normal epidermis: a missing link in the desquamation process? *J Invest Dermatol* 114:56–63

Hachem JP, Houben E, Crumrine D *et al.* (2006) Serine protease signaling of epidermal permeability barrier homeostasis. *J Invest Dermatol* 126: 2074–86

Harrison WJ, Bull JJ, Seltmann H *et al.* (2007) Expression of lipogenic factors galectin-12, resistin, SREBP-1, and SCD in human sebaceous glands and cultured sebocytes. *J Invest Dermatol* 127:1309–17

Iwata C, Akimoto N, Sato T *et al.* (2005) Augmentation of lipogenesis by 15-deoxy-Delta12,14-prostaglandin J2 in hamster sebaceous glands: identification of cytochrome P-450-mediated 15-deoxy-Delta12,14-prostaglandin J2 production. *J Invest Dermatol* 125:865–72

Kang S, Cho S, Chung JH *et al.* (2005) Inflammation and extracellular matrix degradation mediated by activated transcription factors nuclear factor kappaB and activator protein-1 in inflammatory acne lesions in vivo. *Am J Pathol* 166:1691–9

Komatsu N, Takata M, Otsuki N *et al.* (2003) Expression and localization of tissue kallikrein mRNAs in human epidermis and appendages. *J Invest Dermatol* 121:542–9

Komatsu N, Saijoh K, Toyama T *et al.* (2005) Multiple tissue kallikrein mRNA and protein expression in normal skin and skin diseases. *Br J Dermatol* 153:274–81

Lee SE, Kim JM, Jeong SK *et al.* (2010) Protease-activated receptor-2 mediates the expression of inflammatory cytokines, antimicrobial peptides, and matrix metalloproteinases in keratinocytes in response to Propionibacterium acnes. *Arch Dermatol Res* 302:745–56

Makrantonaki E, Ganceviciene R, Zouboulis C. (2011) An update on the role of the sebaceous gland in the pathogenesis of acne. *Dermatoendocrinol* 3: 41–9

Meyer-Hoffert U (2009) Reddish, scaly, and itchy: how proteases and their inhibitors contribute to inflammatory skin diseases. *Arch Immunol Ther Exp* 57:345–54

Moormann C, Artuc M, Pohl E *et al.* (2006) Functional characterization and expression analysis of the proteinase-activated receptor-2 in human cutaneous mast cells. *J Invest Dermatol* 126:746–55

Nagy I, Pivarcsi A, Kis K *et al.* (2006) Propionibacterium acnes and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. *Microbes Infect* 8:2195–205

Nakatsuji T, Kao MC, Zhang L *et al.* (2010) Sebum free fatty acids enhance the innate immune defense of human sebocytes by upregulating beta-defensin-2 expression. *J Invest Dermatol* 130:985–94

Rattenholl A, Steinhoff M (2003) Role of proteinase-activated receptors in cutaneous biology and disease. *Drug Dev Res* 59:408–16

Rattenholl A, Steinhoff M (2008) Proteinase-activated receptor-2 in the skin: receptor expression, activation and function during health and disease. *Drug News Perspect* 21:369–81

Rosignoli C, Nicolas JC, Jomard A *et al.* (2003) Involvement of the SREBP pathway in the mode of action of androgens in sebaceous glands in vivo. *Exp Dermatol* 12:480–9

Smith TM, Gilliland K, Clawson GA *et al.* (2008) IGF-1 induces SREBP-1 expression and lipogenesis in SEB-1 sebocytes via activation of the phosphoinositide 3-kinase/Akt pathway. *J Invest Dermatol* 128: 1286–93

Steinhoff M, Corvera CU, Thoma MS *et al.* (1999) Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Exp Dermatol* 8:282–94

Steinhoff M, Vergnolle N, Young SH *et al.* (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* 6:151–8

Steinhoff M, Buddenkotte J, Shpacovitch V *et al.* (2005) Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. *Endocr Rev* 26:1–43

Toyoda M, Morohashi M (2003) New aspects in acne inflammation. *Dermatology* 206:17–23

Uehara A, Muramoto K, Takada H *et al.* (2003) Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. *J Immunol* 170:5690–6

Wróbel A, Seltmann H, Fimmel S *et al.* (2003) Differentiation and apoptosis in human immortalized sebocytes. *J Invest Dermatol* 120:175–81

Zhang L, Li WH, Anthonavage M *et al.* (2006) Melanocortin-5 receptor: a marker of human sebocyte differentiation. *Peptides* 27:413–20

Zouboulis CC (2009) Propionibacterium acnes and sebaceous lipogenesis: a love-hate relationship? *J Invest Dermatol* 129:2093–6

Zouboulis CC, Schagen S, Alestas T (2008) The sebocyte culture-a model to study the pathophysiology of the sebaceous gland in sebostasis, seborrhoea and acne. *Arch Dermatol Res* 300:397–413

Zouboulis CC, Seltmann H, Neitzel H *et al.* (1999) Establishment and characterization of an immortalized human sebaceous gland cell line (SZ95). *J Invest Dermatol* 113:1011–20